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(54) Title: TREATING GRAFT REJECTION WITH CCR5 INHIBITORS

(57) Abstract: A method for inhibiting the rejection of transplanted grafts is disclosed. The method comprising administering an effective amount of an antagonist of CCR5 function to a graft recipient. The disclosed methods can also comprise the co-administration of one or more additional therapeutic agents, for example, immunosuppressive agents.

TREATING GRAFT REJECTION WITH CCR5 INHIBITORS

BACKGROUND OF THE INVENTION

In many instances, the best and only treatment available to patients suffering from certain end stage degenerative conditions or congenital genetic disorders is transplantation of a healthy graft (e.g., organs, tissues). Advances in surgical techniques and post-operative immunosuppressive therapy have mitigated some of the barriers to long-term survival of grafts and graft recipients, and ushered this once experimental therapy into wider clinical practice.

A major barrier to the long-term survival of transplanted grafts is rejection by the recipient's immune system. Graft rejection can be classified as hyper-acute rejection which is mediated by preformed antibodies that can bind to the graft and are present in the circulation of the recipient, acute rejection which is mediated by the recipient's cellular immune response or chronic rejection which occurs via a multi-factorial process that includes an immune component. The practice of matching the allelic variants of cellular antigens, most notably major histocompatibility antigens (MHC), also referred to as tissue typing, as well as matching of the blood type of the donor and recipient has reduced the incidence of hyper-acute rejection. However, most grafts which are transplanted do not exactly match the tissue type of the recipient (e.g., allografts) and will not remain viable without therapeutic intervention.

The rejection of allografts can be inhibited by long-term (e.g., life-long) prophylactic immunosuppressive therapy, most notably with agents that inhibit calcineurin (e.g., cyclosporin A (CsA), FK-506). Immunosuppressive therapy not only inhibits rejection of the graft, but can render the recipient susceptible to infection with, for example, viruses, bacteria and fungi (e.g., yeasts, molds), and at higher risk for the development of certain malignancies. Additionally, therapeutic doses of immunosuppressive agents can produce adverse side effects, such as diabetes mellitus, neurotoxicity, nephrotoxicity, hyperlipidemia, hypertension, hirsutism and gingival hyperplasia (Spencer, C.M., *et al.*, *Drugs 54*(6):925-975 (1997)). Thus, the degree of immunosuppression must be carefully tailored to prevent rejection of the graft and to preserve the general health of the recipient.

Despite such prophylactic immunosuppression, acute and chronic rejection of grafts remains a clinical problem. Acute episodes of rejection are characterized by infiltration of the graft by the recipient's leukocytes (e.g., monocytes, macrophages, T cells) and cellular necrosis. These episodes usually occur during the days to months following transplantation. Acute rejection has been treated with high doses of certain immunosuppressive agents, such as glucocorticoids (e.g., prednisone) and certain antibodies which bind to leukocytes (e.g., OKT3). However, these therapies do not always stop the rejection, are associated with systemic side effects and can lose efficacy in cases of recurrent rejection activity.

Chronic rejection becomes the major cause of graft failure and recipient death for those patients that survive past the first year. For example, evidence of chronic rejection can be found in about 40-50% of heart and/or lung allograft recipients who survive for five years, and most kidney grafts succumb to chronic rejection. The pathogenesis of chronic rejection is complex and involves accelerated arteriosclerosis (e.g., atherosclerosis) of the graft-associated vasculature and leukocyte infiltration. Unlike acute rejection episodes, chronic rejection is not generally responsive to further immunosuppressive therapy. Furthermore, the graft accelerated arteriosclerosis characteristic of chronic rejection is generally diffuse and not amenable to conventional therapeutic procedures (e.g., angioplasty, bypass grafting, endarterectomy). Thus, patients who chronically reject their grafts can

require a second transplant (Schroeder J.S. "Cardiac Transplantation", pp. 1298-1300; Maurer, J.R. "Lung Transplantation", pp. 1491-1493; Carpenter, C.B. and Lazarus, J.M. "Dialysis and Transplantation in the Treatment of Renal Failure", pp. 1524-1529; Dienstag, J. "Liver Transplantation", pp. 1721-1725; all in *Harrison's Principles of Internal Medicine*, 14th ed., Fauci *et al.* Eds. McGraw Hill (1998)).

A need exists for therapeutic methods for preventing graft rejection.

SUMMARY OF THE INVENTION

The invention relates to transplantation and to promoting the viability of transplanted grafts. In one aspect, the invention relates to a method for inhibiting (reducing or preventing) graft rejection (e.g., acute rejection, chronic rejection). In one embodiment, the method comprises administering to a graft recipient an effective amount of an antagonist of CCR5 function. In another embodiment, the graft is an allograft. In a particular embodiment, the allograft is a heart. In a preferred embodiment, the method comprises administration of an effective amount of an antagonist of CCR5 function and an effective amount of one or more immunosuppressive agents to a graft recipient.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to transplantation and to promoting the viability of transplanted grafts. Specifically, the invention relates to inhibiting graft rejection (e.g., acute graft rejection, chronic graft rejection) by administering to a graft recipient an effective amount of an antagonist of mammalian (e.g., human, *Homo sapiens*) CC chemokine receptor 5, CCR5.

Chemokines are a family of proinflammatory mediators that promote recruitment and activation of multiple lineages of leukocytes (e.g., lymphocytes, macrophages). They can be released by many kinds of tissue cells after activation. Continuous release of chemokines at sites of inflammation can mediate the ongoing migration and recruitment of effector cells to sites of chronic inflammation. The chemokines are related in primary structure and share four conserved cysteines, which form disulfide bonds. Based upon this conserved cysteine motif, the family

can be divided into distinct branches, including the C-X-C chemokines (α-chemokines), and the C-C chemokines (β-chemokines), in which the first two conserved cysteines are separated by an intervening residue, or are adjacent residues, respectively (Baggiolini, M. and Dahinden, C. A., *Immunology Today*, *15*:127-133 (1994)).

The C-X-C chemokines include a number of potent chemoattractants and activators of neutrophils, such as interleukin 8 (IL-8), PF4 and neutrophil-activating peptide-2 (NAP-2). The C-C chemokines include, for example, RANTES (Regulated on Activation, Normal T Expressed and Secreted), macrophage inflammatory proteins 1α and 1β (MIP-1α and MIP-1β), eotaxin and human monocyte chemotactic proteins 1-3 (MCP-1, MCP-2, MCP-3), which have been characterized as chemoattractants and activators of monocytes or lymphocytes. Chemokines, such as IL-8, RANTES and MIP-1α, for example, have been implicated in human acute and chronic inflammatory diseases including respiratory diseases, such as asthma and allergic disorders.

The chemokine receptors are members of a superfamily of G protein-coupled receptors (GPCR) which share structural features that reflect a common mechanism of action of signal transduction (Gerard, C. and Gerard, N.P., *Annu Rev. Immunol.*, *12*:775-808 (1994); Gerard, C. and Gerard, N. P., *Curr. Opin. Immunol.*, *6*:140-145 (1994)). Conserved features include seven hydrophobic domains spanning the plasma membrane, which are connected by hydrophilic extracellular and intracellular loops. The majority of the primary sequence homology occurs in the hydrophobic transmembrane regions with the hydrophilic regions being more diverse. The receptors for the C-C chemokines include: CCR1 which can bind, for example, MIP-1α, RANTES, MCP-2, MCP-3, MCP-4, CKbeta8, CKbeta8-1, leukotactin-1, HCC-1 and MPIF-1; CCR2 which can bind, for example, MCP-1, MCP-2, MCP-3 and MCP-4; CCR3 which can bind, for example, eotaxin, eotaxin-2, RANTES, MCP-2, MCP-3 and MCP-4; CCR4 which can bind, for example, TARC, RANTES, MIP-1α and MCP-1; CCR5 which can bind, for example, MIP-1α, RANTES, MIP-1β, MCP-1, MCP-2 and MCP-4; CCR6 which can bind, for example, LARC/MIP-3α/exodus; CCR7 which can bind, for example, ELC/MIP-3β;

CCR8 which can bind, for example, I-309; CCR9 which can bind, for example, TECK and CCR10 which can bind, for example, ESkine and CCL27 (Baggiolini, M., *Nature 392*:565-568 (1998); Luster, A.D., *New England Journal of Medicine, 338*(7):436-445 (1998); Tsou, *et al., J. Exp. Med., 188*:603-608 (1998); Nardelli, *et al., J Immunol, 162*(1):435-444 (1999); Youn, *et al., Blood, 91*(9):3118-3126 (1998); Youn, *et al., J Immunol, 159*(11):5201-5205 (1997); Zaballos, *et al., J Immunol, 162*:5671-5675 (1999); Jarmin, *et al., J Immunol, 164*:3460-3464 (2000); Homey *et al., J Immunol, 164*:3465-3470 (2000)). The receptors for the CXC chemokines include: CXCR1 which can bind, for example, IL-8, GCP-2; CXCR2 which can bind, for example, IL-8, GROα/β/γ, NAP-2, ENA78, GCP-2; CXCR3 which can bind, for example, interferon gamma (IFNγ)-inducible protein of 10kDa (IP-10), monokine induced by IFNγ (Mig), interferon-inducible T cell chemoattractant (I-TAC); CXCR4 which can bind, for example, SDF-1; and CXCR5 which can bind, for example, BCA-1/BLC (Baggiolini M., *Nature, 392*:565-568 (1998); Lu *et al., Eur J Immunol, 29*:3804-3812 (1999)).

CCR5, as well as processes and cellular responses mediated by CCR5, are involved in rejection of transplanted grafts. As described herein, studies of allograft survival using a murine cardiac transplantation model were undertaken. Mice which lacked functional chemokine receptor CCR5 as a result of targeted disruption of the CCR5 gene (CCR5 KO mice) did not reject transplanted allografts, which were mismatched at MHC class I and MHC class II, as rapidly as control mice (CCR5 +/+ mice) which had a functional CCR5 gene and were otherwise genetically identical to CCR5 KO mice (Table 1, groups 1 and 2). In addition, the period of allograft survival in wild type recipient mice was significantly extended by treating the recipient with a neutralizing mAb which binds CCR5 and inhibits the binding of ligand to the receptor (Table 1, group 8 and Table 2, group 10). In further studies, allografts that were mismatched only at MHC class II were transplanted into CCR5 KO and CCR5+/+ control mice. As expected, the partial tissue match led to prolonged survival of the graft in CCR5+/+ control mice. However, all CCR5+/+ control mice still rejected the graft by about the thirty-fifth day (Table 1, group 5).

In contrast, partial matching of MHC with inhibition of CCR5 function was highly efficacious and led to permanent (>75 days) engraftment (Table 1, group 6).

Accordingly, a first aspect of the invention provides a method for inhibiting rejection (e.g., acute and/or chronic rejection) of a graft, comprising administering to a graft recipient an effective amount of an antagonist of CCR5 function.

CCR5 antagonists

As used herein, the term "antagonist of CCR5 function" refers to an agent (e.g., a molecule, a compound) which can inhibit a (i.e., one or more) function of CCR5. For example, an antagonist of CCR5 function can inhibit the binding of one or more ligands (e.g., MIP-1α, RANTES, MIP-1β) to CCR5 and/or inhibit signal transduction mediated through CCR5 (e.g., GDP/GTP exchange by CCR5-associated G proteins, intracellular calcium flux). Accordingly, CCR5-mediated processes and cellular responses (e.g., proliferation, migration, chemotactic responses, secretion or degranulation) can be inhibited with an antagonist of CCR5 function. As used herein, "CCR5" refers to naturally occurring CC chemokine receptor 5 (e.g., mammalian CCR5 (e.g., human (*Homo sapiens*) CCR5)) and encompasses naturally occurring variants, such as allelic variants and splice variants.

Preferably, the antagonist of CCR5 function is a compound which is, for example, a small organic molecule, natural product, protein (e.g., antibody, chemokine, cytokine), peptide or peptidomimetic. Several molecules that can antagonize one or more functions of chemokine receptors (e.g., CCR5) are known in the art, including the small organic molecules disclosed in, for example, international patent application WO 97/24325 by Takeda Chemical Industries, Ltd.; WO 98/38167 by Pfizer, Inc.; WO 97/44329 by Teijin Limited; WO 98/04554 by Banyu Pharmaceutical Co., Ltd.; WO 98/27815, WO 98/25604, WO 98/25605, WO 98/25617 and WO 98/31364 by Merck & Co., Inc.; Hesselgesser *et al., J. Biol. Chem. 273*(25):15687-15692 (1998); and Howard *et al., J. Medicinal Chem. 41*(13):2184-2193 (1998); proteins, such as antibodies (e.g., polyclonal sera, monoclonal, chimeric, humanized, human) and antigen-binding fragments thereof (e.g., Fab, Fab', $F(ab')_2$, Fv), for example, those disclosed in WO 98/18826 by

LeukoSite, Inc.; chemokine mutants and analogues, for example, those disclosed in U.S. Patent No. 5,739,103 issued to Rollins *et al.*, WO 96/38559 by Dana Farber Cancer Institute and WO 98/06751 by Research Corporation Technologies, Inc.; peptides, for example, those disclosed in WO 98/09642 by The United States of America. The entire teachings of each of the above cited patents, patent applications and references are incorporated herein by reference.

Antagonists of CCR5 function can be identified, for example, by screening libraries or collections of molecules, such as, the Chemical Repository of the National Cancer Institute, as described herein or using other suitable methods.

Another source of antagonists of CCR5 function are combinatorial libraries which can comprise many structurally distinct molecular species. Combinatorial libraries can be used to identify lead compounds or to optimize a previously identified lead. Such libraries can be manufactured by well-known methods of combinatorial chemistry and screened by suitable methods, such as the methods described herein.

The term "natural product", as used herein, refers to a compound which can be found in nature, for example, naturally occurring metabolites of marine organisms (e.g., tunicates, algae), plants or other organisms, and which possesses biological activity, e.g., can antagonize CCR5 function. For example, lactacystin, paclitaxel and cyclosporin A are natural products which can be used as anti-proliferative or immunosuppressive agents.

Natural products can be isolated and identified by suitable means. For example, a suitable biological source (e.g., vegetation) can be homogenized (e.g., by grinding) in a suitable buffer and clarified by centrifugation, thereby producing an extract. The resulting extract can be assayed for the capacity to antagonize CCR5 function, for example, by the assays described herein. Extracts which contain an activity that antagonizes CCR5 function can be further processed to isolate the CCR5 antagonist by suitable methods, such as, fractionation (e.g., column chromatography (e.g., ion exchange, reverse phase, affinity), phase partitioning, fractional crystallization) and assaying for biological activity (e.g., antagonism of CCR5 activity). Once isolated the structure of a natural product can be determined

(e.g., by nuclear magnetic resonance (NMR)) and those of skill in the art can devise a synthetic scheme for synthesizing the natural product. Thus, a natural product can be isolated (e.g., substantially purified) from nature or can be fully or partially synthetic. A natural product can be modified (e.g., derivatized) to optimize its therapeutic potential. Thus, the term "natural product", as used herein, includes those compounds which are produced using standard medicinal chemistry techniques to optimize the therapeutic potential of a compound which can be isolated from nature.

The term "peptide", as used herein, refers to a compound consisting of from about two to about ninety amino acid residues wherein the amino group of one amino acid is linked to the carboxyl group of another amino acid by a peptide bond. A peptide can be, for example, derived or removed from a native protein by enzymatic or chemical cleavage, or can be prepared using conventional peptide synthesis techniques (e.g., solid phase synthesis) or molecular biology techniques (see Sambrook, J. *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). A "peptide" can comprise any suitable L- and/or D-amino acid, for example, common α-amino acids (e.g., alanine, glycine, valine), non-α-amino acids (e.g., β-alanine, 4-aminobutyric acid, 6-aminocaproic acid, sarcosine, statine), and unusual amino acids (e.g., citrulline, homocitruline, homoserine, norleucine, norvaline, ornithine). The amino, carboxyl and/or other functional groups on a peptide can be free (e.g., unmodified) or protected with a suitable protecting group. Suitable protecting groups for amino and carboxyl groups, and means for adding or removing protecting groups are known in the art and are disclosed in, for example, Green and Wuts, *"Protecting Groups in Organic Synthesis"*, John Wiley and Sons, 1991. The functional groups of a peptide can also be derivatized (e.g., alkylated) using art-known methods.

Peptides can be synthesized and assembled into libraries comprising a few to many discrete molecular species. Such libraries can be prepared using well-known methods of combinatorial chemistry, and can be screened as described herein or using other suitable methods to determine if the library comprises peptides which

can antagonize CCR5 function. Such peptide antagonists can then be isolated by suitable methods.

The term "peptidomimetic", as used herein, refers to molecules which are not polypeptides, but which mimic aspects of their structures. For example, polysaccharides can be prepared that have the same functional groups as peptides which can antagonize CCR5. Peptidomimetics can be designed, for example, by establishing the three dimensional structure of a peptide agent in the environment in which it is bound or will bind to CCR5. The peptidomimetic comprises at least two components, the binding moiety or moieties and the backbone or supporting structure.

The binding moieties are the chemical atoms or groups which will react or form a complex (e.g., through hydrophobic or ionic interactions) with CCR5, for example, with the amino acid(s) at or near the ligand binding site. For example, the binding moieties in a peptidomimetic can be the same as those in a peptide antagonist of CCR5. The binding moieties can be an atom or chemical group which reacts with the receptor in the same or similar manner as the binding moiety in a peptide antagonist of CCR5. Examples of binding moieties suitable for use in designing a peptidomimetic for a basic amino acid in a peptide are nitrogen containing groups, such as amines, ammoniums, guanidines and amides or phosphoniums. Examples of binding moieties suitable for use in designing a peptidomimetic for an acidic amino acid can be, for example, carboxyl, lower alkyl carboxylic acid ester, sulfonic acid, a lower alkyl sulfonic acid ester or a phosphorous acid or ester thereof.

The supporting structure is the chemical entity that, when bound to the binding moiety or moieties, provides the three dimensional configuration of the peptidomimetic. The supporting structure can be organic or inorganic. Examples of organic supporting structures include polysaccharides, polymers or oligomers of organic synthetic polymers (such as, polyvinyl alcohol or polylactide). It is preferred that the supporting structure possess substantially the same size and dimensions as the peptide backbone or supporting structure. This can be determined by calculating or measuring the size of the atoms and bonds of the peptide and peptidomimetic. In

one embodiment, the nitrogen of the peptide bond can be substituted with oxygen or sulfur, thereby forming a polyester backbone. In another embodiment, the carbonyl can be substituted with a sulfonyl group or sulfinyl group, thereby forming a polyamide (e.g., a polysulfonamide). Reverse amides of the peptide can be made (e.g., substituting one or more -CONH- groups for a -NHCO- group). In yet another embodiment, the peptide backbone can be substituted with a polysilane backbone.

These compounds can be manufactured by known methods. For example, a polyester peptidomimetic can be prepared by substituting a hydroxyl group for the corresponding α-amino group on amino acids, thereby preparing a hydroxyacid and sequentially esterifying the hydroxyacids, optionally blocking the basic and acidic side chains to minimize side reactions. An appropriate chemical synthesis route can generally be readily identified upon determining the desired chemical structure of the peptidomimetic.

Peptidomimetics can be synthesized and assembled into libraries comprising a few to many discrete molecular species. Such libraries can be prepared using well-known methods of combinatorial chemistry, and can be screened as described herein to determine if the library comprises one or more peptidomimetics which antagonize CCR5 function. Such peptidomimetic antagonists can then be isolated by suitable methods.

In one embodiment, the CCR5 antagonist is an antibody or antigen-binding fragment thereof having specificity for CCR5. The antibody can be polyclonal or monoclonal, and the term "antibody" is intended to encompass both polyclonal and monoclonal antibodies. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to particular methods of production. The term "antibody" as used herein also encompasses functional fragments of antibodies, including fragments of chimeric, humanized, primatized, veneered or single-chain antibodies. Functional fragments include antigen-binding fragments which bind to CCR5. For example, antibody fragments capable of binding to CCR5 or portions thereof, including, but not limited to Fv, Fab, Fab' and $F(ab')_2$ fragments can be used. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For example, papain

or pepsin cleavage can generate Fab or $F(ab')_2$ fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or $F(ab')_2$ fragments. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a chimeric gene encoding a $F(ab')_2$ heavy chain portion can be designed to include DNA sequences encoding the CH_1 domain and hinge region of the heavy chain. Single-chain antibodies, and chimeric, human, humanized or primatized (CDR-grafted), or veneered antibodies, as well as chimeric, CDR-grafted or veneered single-chain antibodies, comprising portions derived from different species, and the like are also encompassed by the present invention and the term "antibody". The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S. Patent No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M.S. *et al.*, WO 86/01533; Neuberger, M.S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, European Patent No. 0 451 216 B1; and Padlan, E.A. *et al.*, EP 0 519 596 A1. See also, Newman, R. *et al., BioTechnology, 10:* 1455-1460 (1992), regarding primatized antibody, and Ladner *et al.*, U.S. Patent No. 4,946,778 and Bird, R.E. *et al., Science, 242:* 423-426 (1988)) regarding single-chain antibodies.

Humanized antibodies can be produced using synthetic or recombinant DNA technology using standard methods or other suitable techniques. Nucleic acid (e.g., cDNA) sequences coding for humanized variable regions can also be constructed using PCR mutagenesis methods to alter DNA sequences encoding a human or humanized chain, such as a DNA template from a previously humanized variable region (see e.g., Kamman, M., *et al., Nucl. Acids Res., 17*: 5404 (1989)); Sato, K., *et al., Cancer Research, 53*: 851-856 (1993); Daugherty, B.L. *et al., Nucleic Acids Res., 19(9):* 2471-2476 (1991); and Lewis, A.P. and J.S. Crowe, *Gene, 101*: 297-302

(1991)). Using these or other suitable methods, variants can also be readily produced. In one embodiment, cloned variable regions can be mutated, and sequences encoding variants with the desired specificity can be selected (e.g., from a phage library; see e.g., Krebber *et al.*, U.S. 5,514,548; Hoogenboom *et al.*, WO 93/06213, published April 1, 1993).

Antibodies which are specific for mammalian (e.g., human) CCR5 can be raised against an appropriate immunogen, such as isolated and/or recombinant human CCR5 or portions thereof (including synthetic molecules, such as synthetic peptides). Antibodies can also be raised by immunizing a suitable host (e.g., mouse, rat) with cells that express CCR5, such as activated T cells (see, e.g., U.S. Pat. No. 5,440,020, the entire teachings of which are incorporated herein by reference). In addition, cells expressing recombinant CCR5 such as transfected cells, can be used as immunogens or in a screen for antibody which binds receptor (see, e.g., Chuntharapai *et al., J. Immunol., 152:* 1783-1789 (1994); Chuntharapai *et al.*; U.S. Patent No. 5,440,021).

Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed using any suitable technique. A variety of methods have been described (see e.g., Kohler *et al., Nature, 256:* 495-497 (1975) and *Eur. J. Immunol. 6:* 511-519 (1976); Milstein *et al., Nature 266: 550-552 (1977);* Koprowski *et al.*, U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual,* (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. *et al.*, Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). When a monoclonal antibody is desired, a hybridoma can generally be produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0 or P3X63Ag8.653) with antibody-producing cells. The antibody-producing cells, preferably those obtained from the spleen or lymph nodes, can be obtained from animals immunized with the antigen of interest. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, for example, methods which select recombinant antibody from a library (e.g., a phage display library). Transgenic animals capable of producing a repertoire of human antibodies (e.g., XenoMouse™ (Abgenix, Fremont, CA)) can be produced using suitable methods (see, e.g., WO 98/24893 (Abgenix), published June 11, 1998; Kucherlapati, R. and Jakobovits, A., U.S. Patent No. 5,939,598; Jakobovits *et al., Proc. Natl. Acad. Sci. USA, 90*: 2551-2555 (1993); Jakobovits *et al., Nature, 362*: 255-258 (1993)). Additional methods for production of transgenic animals capable of producing a repertoire of human antibodies have been described (e.g., Lonberg *et al.*, U.S. Patent No. 5,545,806; Surani *et al.*, U.S. Patent No. 5,545,807; Lonberg *et al.*, WO97/13852).

In one embodiment, the antibody or antigen-binding fragment thereof has specificity for a mammalian CC chemokine receptor 5 (CCR5), such as human CCR5. In a preferred embodiment, the antibody or antigen-binding fragment can inhibit binding of a ligand (i.e., one or more ligands) to CCR5 and/or one or more functions mediated by CCR5 in response to ligand binding. Preferred antibody antagonists of CCR5 function, such as murine mAb 5C7 and murine mAb 2D7 are disclosed in WO 98/18826 published May 7, 1998, and United States Patent Application No. 08/893,911, filed July 11, 1997, the teachings of both of which are incorporated herein by reference in their entirety. These antibodies and, for example, chimeric or humanized versions of these antibodies, can be administered in accordance with the method of the invention.

The 5C7 hybridoma cell line (also referred to as LS87 5C7) which produces murine mAb 5C7 was deposited on October 25, 1996 on behalf of LeukoSite, Inc., 215 First Street, Cambridge, MA 02142 (now Millennium Pharmaceuticals, Inc., 75 Sidney Street, Cambridge, MA 02139), under the terms of the Budapest Treaty at the American Type Culture Collection, 10801 University Boulevard, Manassas, VA, 20110, under Accession Number HB-12222.

The 2D7 hybridoma cell line (also referred to as 2D7 LS100-2D7-13-1-1-14-14-4) which produces murine mAb 2D7 was deposited on June 6, 1997, on behalf of LeukoSite, Inc., 215 First Street, Cambridge, MA 02142 (now Millennium

Pharmaceuticals, Inc., 75 Sidney Street, Cambridge, MA 02139), under the terms of the Budapest Treaty at the American Type Culture Collection, 10801 University Boulevard, Manassas, VA, 20110, under Accession Number HB-12366.

Antibodies which bind CCR5 ligand (e.g., MIP-1α, RANTES, MIP-1β, MCP-1, MCP-2, MCP-4) and inhibit binding of ligand to CCR5 can be prepared using suitable method, such as the methods described herein.

Assessment of Activity of Antagonists

The capacity of an agent (e.g., proteins, peptides, natural products, small organic molecules, peptidomimetics) to antagonize CCR5 function can be determined using a suitable screen (e.g., high through-put assay). For example, an agent can be tested in an extracellular acidification assay, calcium flux assay, ligand binding assay or chemotaxis assay (see, for example, Hesselgesser *et al., J. Biol. Chem. 273(25)*:15687-15692 (1998); WO 98/18826 and WO 98/02151).

In a particular assay, membranes can be prepared from cells which express CCR5, such as activated T cells or cells which express recombinant CCR5. Cells can be harvested by centrifugation, washed twice with PBS (phosphate-buffered saline), and the resulting cell pellets frozen at -70 to -85°C. The frozen pellet can be thawed in ice-cold lysis buffer consisting of 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) pH 7.5, 2 mM EDTA (ethylenediaminetetraacetic acid), 5 μg/ml each aprotinin, leupeptin, and chymostatin (protease inhibitors), and 100 μg/ml PMSF (phenyl methane sulfonyl fluoride - also a protease inhibitor), at a concentration of 1 to 5 x 10^7 cells/ml, to achieve cell lysis. The resulting suspension can be mixed well to resuspend all of the frozen cell pellet. Nuclei and cell debris can be removed by centrifugation at 400 x g for 10 minutes at 4°C. The resulting supernatant can be transferred to a fresh tube and the membrane fragments can be collected by centrifugation at 25,000 x g for 30 minutes at 4°C. The resulting supernatant can be aspirated and the pellet can be resuspended in freezing buffer consisting of 10 mM HEPES pH 7.5, 300 mM sucrose, 1μg/ml each aprotinin, leupeptin, and chymostatin, and 10 μg/ml PMSF (approximately 0.1 ml per each 10^8 cells). All clumps can be resolved using a

minihomogenizer, and the total protein concentration can be determined by suitable methods (e.g., Bradford assay, Lowery assay). The membrane solution can be divided into aliquots and frozen at -70 to -85°C until needed.

The membrane preparation described above can be used in a suitable binding assay. For example, membrane protein (2 to 20 μg total membrane protein) can be incubated with 0.1 to 0.2 nM ^{125}I-labeled MIP-1α, ^{125}I-labeled RANTES or ^{125}I-labeled MIP-1β with or without unlabeled competitor (e.g., MIP-1α, RANTES and/or MIP-1β) or various concentrations of compounds to be tested. ^{125}I-labeled chemokines (e.g., MIP-1α, RANTES, MIP-1β) can be prepared by suitable methods or purchased from commercial vendors (e.g., DuPont-NEN (Boston, MA)). The binding reactions can be performed in 60 to 100 μl of binding buffer consisting of 10 mM HEPES pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% BSA (bovine serum albumin), for 60 min at room temperature. The binding reactions can be terminated by harvesting the membranes by rapid filtration through glass fiber filters (e.g., GF/B or GF/C, Packard) which can be presoaked in 0.3% polyethyleneimine. The filters can be rinsed with approximately 600 μl of binding buffer containing 0.5 M NaCl, dried, and the amount of bound radioactivity can be determined by scintillation counting.

The CCR5 antagonist activity of test agents (e.g., compounds) can be reported as the inhibitor concentration required for 50% inhibition (IC_{50} values) of specific binding in receptor binding assays (e.g., using ^{125}I-MIP-1α, ^{125}I-RANTES or ^{125}I-MIP-1β as ligand and membranes prepared from activated T cells). Specific binding is preferably defined as the total binding (e.g., total cpm on filters) minus the non-specific binding. Non-specific binding is defined as the amount of cpm still detected in the presence of excess unlabeled competitor (e.g., MIP-1α, RANTES, MIP-1β). If desired, membranes prepared from cells which express recombinant CCR5 can be used in the described assay.

The capacity of compounds to antagonize CCR5 function can also be determined in a leukocyte chemotaxis assay using suitable cells. Suitable cells include, for example, cell lines, recombinant cells or isolated cells which express CCR5 and undergo CCR5 ligand-induced (e.g., MIP-1α, RANTES, MIP-1β, MCP-

1, MCP-2, MCP-4) chemotaxis. In one example, CCR5-expressing recombinant L1.2 cells (see, e.g., Campbell *et al. J Cell Biol, 134*:255-266 (1996)) or activated T cells, can be used in a modification of a transendothelial migration assay (Carr, M.W., *et al., Proc. Natl Acad Sci, USA, (91)*:3652 (1994)). T cells can be isolated from whole blood by suitable methods, for example, density gradient centrifugation and positive or preferably negative selection with specific antibodies and activated using, for example, mitogens, anti-CD3 or cytokines (e.g., IL-2). The endothelial cells used in this assay are preferably the endothelial cell line, ECV 304, obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.). Endothelial cells can be cultured on 6.5 mm diameter Transwell culture inserts (Costar Corp., Cambridge, MA) with 3.0 μm pore size. Culture media for the ECV 304 cells can consist of M199 + 10% FCS, L-glutamine, and antibiotics. The assay media can consist of equal parts RPMI 1640 and M199 with 0.5% BSA. Two hours before the assay, $2x10^5$ ECV 304 cells can be plated onto each insert of the 24-well Transwell chemotaxis plate and incubated at 37°C. Chemotactic factors such as MIP-1α, RANTES or MIP-1β (commercially available from Peprotech, Rocky Hill, NJ, for example) diluted in assay medium can be added to the 24-well tissue culture plates in a final volume of 600 μL. Endothelial-coated Transwells can be inserted into each well and 10^6 cells of the leukocyte type being studied are added to the top chamber in a final volume of 100 μL of assay medium. The plate can then be incubated at 37°C in 5% CO_2/95% air for 1-2 hours. The cells that migrate to the bottom chamber during incubation can be counted, for example using flow cytometry. To count cells by flow cytometry, 500 μL of the cell suspension from the lower chamber can be placed in a tube and relative counts can be obtained for a set period of time, for example, 30 seconds. This counting method is highly reproducible and allows gating on the leukocytes and the exclusion of debris or other cell types from the analysis. Alternatively, cells can be counted with a microscope. Assays to evaluate chemotaxis inhibitors can be performed in the same way as control experiment described above, except that antagonist solutions, in assay media containing up to 1% of DMSO co-solvent, can be added to both the top and bottom chambers prior to addition of the cells. Antagonist potency can be determined by

comparing the number of cells that migrate to the bottom chamber in wells which contain antagonist, to the number of cells which migrate to the bottom chamber in control wells. Control wells can contain equivalent amounts of DMSO, but no antagonist. If desired, the endothelial cells can be omitted from the described chemotaxis assay and ligand-induced migration across the Transwell insert can be measured.

The activity of an antagonist of CCR5 function can also be assessed by monitoring cellular responses induced by active receptor, using suitable cells expressing receptor. For instance, exocytosis (e.g., degranulation of cells leading to release of one or more enzymes or other granule components, such as esterases (e.g., serine esterases), perforin, and/or granzymes), inflammatory mediator release (such as release of bioactive lipids such as leukotrienes (e.g., leukotriene C_4)), and respiratory burst, can be monitored by methods known in the art or other suitable methods (see e.g., Taub, D.D. *et al., J. Immunol., 155:* 3877-3888 (1995), regarding assays for release of granule-derived serine esterases; Loetscher *et al., J. Immunol., 156*: 322-327 (1996), regarding assays for enzyme and granzyme release; Rot, A. *et al., J. Exp. Med., 176*: 1489-1495 (1992) regarding respiratory burst; Bischoff, S.C. *et al., Eur. J. Immunol., 23*: 761-767 (1993) and Baggiolini, M. and C.A. Dahinden, *Immunology Today, 15*: 127-133 (1994)).

In one embodiment, an antagonist of CCR5 is identified by monitoring the release of an enzyme upon degranulation or exocytosis by a cell capable of this function. Cells expressing CCR5 can be maintained in a suitable medium under suitable conditions, and degranulation can be induced. The cells are contacted with an agent to be tested, and enzyme release can be assessed. The release of an enzyme into the medium can be detected or measured using a suitable assay, such as in an immunological assay, or biochemical assay for enzyme activity.

The medium can be assayed directly, by introducing components of the assay (e.g., substrate, co-factors, antibody) into the medium (e.g., before, simultaneous with or after the cells and agent are combined). The assay can also be performed on medium which has been separated from the cells or further processed (e.g., fractionated) prior to assay. For example, convenient assays are available for

enzymes, such as serine esterases (see e.g., Taub, D.D. *et al., J. Immunol., 155:* 3877-3888 (1995) regarding release of granule-derived serine esterases).

In another embodiment, cells expressing CCR5 are combined with a ligand of CCR5 or promoter of CCR5 function, an agent to be tested is added before, after or simultaneous therewith, and degranulation is assessed. Inhibition of ligand- or promoter-induced degranulation is indicative that the agent is an inhibitor of mammalian CCR5 function.

In a preferred embodiment, the antagonist of CCR5 function does not significantly inhibit the function of other chemokine receptors (e.g., CCR1, CXCR1, CCR3). Such CCR5-specific antagonists can be identified by suitable methods, such as by suitable modification of the methods described herein. For example, cells which do not express CCR5 ($CCR5^-$) but do express one or more other chemokine receptors (e.g., CCR2, CXCR1, CCR9) can be made or identified using suitable methods (e.g., transfection, antibody staining, western blot, RNAse protection). Such cells or cellular fractions (e.g., membranes) obtained from such cells can be used in a suitable binding assay. For example, when a cell which is $CCR5^-$ and $CCR2^+$ is chosen, the CCR5 antagonist can be assayed for the capacity to inhibit the binding of a suitable CCR2 ligand (e.g., MCP-1) to the cell or cellular fraction, as described herein.

In another preferred embodiment, the antagonist of CCR5 function is an agent which binds CCR5. Such CCR5-binding antagonists can be identified by suitable methods, for example, in binding assays employing a labeled (e.g., enzymatically-labeled (e.g., alkaline phosphatase, horse radish peroxidase), biotinylated, radio-labeled (e.g., ^3H, ^{14}C, ^{125}I)) antagonist.

In another preferred embodiment, the antagonist of CCR5 function is an agent which can inhibit the binding of a (i.e., one or more) CCR5 ligand to CCR5, such as an agent which can inhibit binding of human MIP-1α, RANTES, MIP-1β, MCP-1, MCP-2 and/or MCP-4 to human CCR5.

In a particularly preferred embodiment, the antagonist of CCR5 function is an agent which can bind to CCR5 and thereby inhibit the binding of a (i.e., one or more) CCR5 ligand to CCR5 (e.g., human CCR5).

Methods of Therapy

As used herein, the term "graft" refers to organs and/or tissues which can be obtained from a first mammal (or donor) and transplanted into a second mammal (a recipient), preferably a human. The term "graft" encompasses, for example, skin, eye or portions of the eye (e.g., cornea, retina, lens), muscle, bone marrow or cellular components of the bone marrow (e.g., stem cells, progenitor cells), heart, lung, heart-lung (e.g., heart and a single lung, heart and both lungs), liver, kidney, pancreas (e.g., islet cells, β-cells), parathyroid, bowel (e.g., colon, small intestine, duodenum), neuronal tissue, bone and vasculature (e.g., artery, vein). A graft can be obtained from a suitable mammal (e.g., human, pig, baboon, chimpanzee), or under certain circumstances a graft can be produced *in vitro* by culturing cells, for example, embryonal cells, fetal cells, skin cells, blood cells and bone marrow cells which were obtained from a suitable mammal. A graft is preferably obtained from a human.

The graft can be obtained from a genetically modified animal or can be modified (e.g., genetically, chemically, physically) using any suitable method. In one embodiment, a modified graft having reduced capacity to express a ligand for CCR5 (e.g., MIP-1α, RANTES, MIP-1β, MCP-1, MCP-2, MCP-4), relative to a suitable control (e.g., an unmodified or wild type graft) is transplanted. Such a graft can, for example, carry a targeted mutation in a gene encoding a CCR5 ligand. Targeted mutations can be produced using a variety of suitable methods. For example, a targeted mutation can be introduced into the genome of embryonic stem cells or zygotes using standard techniques. The resulting mutant cells can develop into animals carrying the targeted mutation (e.g., heterozygous or homozygous). For example, pigs or other animals which express human MHC antigens and which are homozygous for a targeted mutation in a gene encoding a CCR5 ligand (e.g., MIP-1α, RANTES, MIP-1β, MCP-1, MCP-2, MCP-4) can be created. The organs from such animals (xenografts) can be transplanted into a human.

An "allograft", as the term is used herein, refers to a graft comprising antigens which are allelic variants of the corresponding antigens found in the recipient. For example, a human graft comprising an MHC class II antigen encoded by the HLA-

DRB1*0401 allele is an allograft if transplanted into a human recipient whose genome does not comprise the HLA-DRB1*0401 allele.

In one embodiment, the method of inhibiting (reducing or preventing) graft rejection comprises administering an effective amount of an (i.e., one or more) antagonist of CCR5 function to a recipient of a graft. In another embodiment, the method of inhibiting graft rejection comprises administering an effective amount of an antagonist of CCR5 function to a recipient of an allograft. In a preferred embodiment, the method comprises administering an effective amount of an antagonist of CCR5 function to a recipient of a cardiac allograft.

In another embodiment, the antagonist of CCR5 function is selected from the group consisting of small organic molecules, natural products, peptides, peptidomimetics and proteins, wherein said proteins are not chemokines or mutants or analogues thereof.

In a preferred embodiment, the invention provides a method for inhibiting (reducing or preventing) graft rejection comprising administering to a graft recipient an effective amount of an antagonist of CCR5 function and an effective amount of an (i.e., one or more) additional therapeutic agent, preferably an immunosuppressive agent. Advantageously, the rejection-inhibiting effects of CCR5 antagonists and immunosuppressive agents can be additive or synergistic, and can result in permanent engraftment.

A further benefit of co-administration of a CCR5 antagonist and an immunosuppressive agent is that the dose of immunosuppressive agent required to inhibit graft rejection can be reduced to sub-therapeutic levels (e.g., a dose that does not inhibit graft rejection when administered as the sole therapeutic agent). The ability to reduce the dose of the immunosuppressive agent can greatly benefit the graft recipient as many immunosuppressive agents have severe and well-known side effects including, for example, increased incidence of infection, increased incidence of certain malignancies, diabetes mellitus, neurotoxicity, nephrotoxicity, hyperlipidemia, hypertension, hirsutism, gingival hyperplasia, impaired wound healing, lymphopenia, jaundice, anemia, alopecia and thrombocytopenia (Spencer,

C.M., *et al.*, *Drugs, 54*(6):925-975 (1997); *Physicians Desk Reference*, 53rd Edition, Medical Economics Co., pp. 2081-2082 (1999)).

The term "immunosuppressive agent", as used herein, refers to compounds which can inhibit an immune response. The immunosuppressive agent used in the invention can be a novel compound or can be selected from the compounds which are known in the art, for example, calcineurin inhibitors (e.g., cyclosporin A, FK-506), IL-2 signal transduction inhibitors (e.g., rapamycin), glucocorticoids (e.g., prednisone, dexamethasone, methylprednisolone, prednisolone), nucleic acid synthesis inhibitors (e.g., azathioprine, mercaptopurine, mycophenolic acid) and antibodies to lymphocytes or antigen-binding fragments thereof (e.g., OKT3, anti-IL2 receptor). Novel immunosuppressive agents can be identified by those of skill in the art using suitable methods, for example, screening compounds for the capacity to inhibit antigen-dependent T cell activation.

The immunosuppressive agent used for co-therapy (e.g., co-administration with an antagonist of CCR5 function) is preferably a calcineurin inhibitor. More preferably the immunosuppressive agent used for co-therapy is cyclosporin A.

When the graft is bone marrow, cells (e.g., leukocytes) derived from the graft can mount an immune response directed at the recipient's organs and tissues. Such a condition is referred to in the art as graft versus host disease (GVHD). Administration of an antagonist of CCR5 function with or without an additional therapeutic agent (e.g., immunosuppressive agent, hematopoietic growth factor) can inhibit GVHD. Accordingly, in another embodiment, the invention provides a method of inhibiting (reducing or preventing) GVHD in a bone marrow graft recipient comprising administering an effective amount of an antagonist of CCR5 function. In an additional embodiment, the method of inhibiting GVHD comprises the administration of an effective amount of an antagonist of CCR5 function and an effective amount of one or more additional therapeutic agents, for example, an immunosuppressive agent.

In another embodiment, the method of inhibiting GVHD comprises the administration of an effective amount of an antagonist of CCR5 function, which is selected from the group consisting of small organic molecules, natural products,

peptides, peptidomimetics and proteins, wherein said proteins are not chemokines or mutants or analogues thereof.

The invention further relates to the use of an antagonist of CCR5 function for the manufacture of a medicament for inhibiting graft rejection (e.g., acute rejection, chronic rejection) as described herein. The invention also relates to a medicament for inhibiting graft rejection (e.g., acute rejection, chronic rejection) wherein said medicament comprises an antagonist of CCR5 function.

A "subject" is preferably a human, but can also be a mammal in need of veterinary treatment, e.g., domestic animals (e.g., dogs, cats, and the like), farm animals (e.g., cows, sheep, fowl, pigs, horses, and the like) and laboratory animals (e.g., rats, mice, guinea pigs, and the like).

An effective amount of the antagonist of CCR5 function can be administered to a subject to inhibit (reduce or prevent) graft rejection. For example, an effective amount of the antagonist of CCR5 function can be administered before, during and/or after transplant surgery or other medical procedure for introduction of a graft to a recipient (e.g., transfusion).

When co-administration of an antagonist of CCR5 function and an additional therapeutic agent is indicated or desired for inhibiting graft rejection, the antagonist of CCR5 function can be administered before, concurrently with or after administration of the additional therapeutic agent. When the antagonist of CCR5 function and additional therapeutic agent are administered at different times, they are preferably administered within a suitable time period to provide substantial overlap of the pharmacological activity (e.g., inhibition of CCR5 function, immunosuppression) of the agents. The skilled artisan will be able to determine the appropriate timing for co-administration of an antagonist of CCR5 function and an additional therapeutic agent depending on the particular agents selected and other factors.

An "effective amount" of a CCR5 antagonist is an amount sufficient to achieve a desired therapeutic and/or prophylactic effect, such as an amount sufficient to inhibit graft rejection. For example, an effective amount is an amount sufficient to inhibit a (i.e., one or more) function of CCR5 (e.g., CCR5 ligand-induced leukocyte

migration, CCR5 ligand-induced integrin activation, CCR5 ligand-induced transient increase in the concentration of intracellular free calcium $[Ca^{2+}]_i$ and/or CCR5 ligand-induced secretion (e.g., degranulation) of proinflammatory mediators), and thereby inhibit graft rejection. An "effective amount" of an additional therapeutic agent (e.g., immunosuppressive agent) is an amount sufficient to achieve a desired therapeutic and/or prophylactic effect (e.g., immunosuppression).

The amount of agent (e.g., CCR5 antagonist, additional therapeutic agent) administered to the individual will depend on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs as well as the degree, severity and type of rejection. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. Typically, an effective amount can range from about 0.1 mg per day to about 100 mg per day for an adult. Preferably, the dosage ranges from about 1 mg per day to about 100 mg per day. Antibodies and antigen-binding fragments thereof, particularly human, humanized and chimeric antibodies and antigen-binding fragments can often be administered less frequently than other types of therapeutics. For example, an effective amount of such an antibody can range from about 0.01 mg/kg to about 5 or 10 mg/kg administered daily, weekly, biweekly, monthly or less frequently.

The agent (e.g., CCR5 antagonist, additional therapeutic agent) can be administered by any suitable route, including, for example, orally (e.g., in capsules, suspensions or tablets) or by parenteral administration. Parenteral administration can include, for example, intramuscular, intravenous, intraarticular, intraarterial, intrathecal, subcutaneous, or intraperitoneal administration. The agent (e.g., CCR5 antagonist, additional therapeutic agent) can also be administered orally (e.g., dietary), transdermally, topically, by inhalation (e.g., intrabronchial, intranasal, oral inhalation or intranasal drops) or rectally. Administration can be local or systemic as indicated. The preferred mode of administration can vary depending upon the particular agent (e.g., CCR5 antagonist, additional therapeutic agent) chosen, however, oral or parenteral administration is generally preferred.

The agent (e.g., CCR5 antagonist, additional therapeutic agent) can be administered as a neutral compound or as a salt. Salts of compounds containing an

amine or other basic group can be obtained, for example, by reacting with a suitable organic or inorganic acid, such as hydrogen chloride, hydrogen bromide, acetic acid, perchloric acid and the like. Compounds with a quaternary ammonium group also contain a counteranion such as chloride, bromide, iodide, acetate, perchlorate and the like. Salts of compounds containing a carboxylic acid or other acidic functional group can be prepared by reacting with a suitable base, for example, a hydroxide base. Salts of acidic functional groups contain a countercation such as sodium, potassium and the like.

The antagonist of CCR5 function can be administered to the individual as part of a pharmaceutical composition for inhibition of graft rejection comprising a CCR5 antagonist and a pharmaceutically or physiologically acceptable carrier. Pharmaceutical compositions for co-therapy can comprise an antagonist of CCR5 function and one or more additional therapeutic agents. An antagonist of CCR5 function and an additional therapeutic agent can be components of separate pharmaceutical compositions which can be mixed together prior to administration or administered separately. Formulation will vary according to the route of administration selected (e.g., solution, emulsion, capsule). Suitable pharmaceutical or physiological carriers can contain inert ingredients which do not interact with the antagonist of CCR5 function and/or additional therapeutic agent. Standard pharmaceutical formulation techniques can be employed, such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. Suitable carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's-lactate and the like. Methods for encapsulating compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art (Baker, *et al.*, "Controlled Release of Biological Active Agents", John Wiley and Sons, 1986).

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

EXAMPLES

Example 1 CCR5 Targeting and Cardiac Transplantation

Methods

Mice. CCR5 KO mice (also referred to as CCR5 -/-) (strain B6/129, H-2^b) which are homozygous for a targeted disruption of the CCR5 gene were obtained from Jackson Laboratory (Bar Harbor, ME). MIP-1α KO mice (also referred to as MIP-1α -/-, strain C57BL/6, H-2^b) which are homozygous for a targeted gene disruption of the gene encoding MIP-1α, RANTES KO mice (also referred to as RANTES -/-, strain C57BL/6, H-2^b) which are homozygous for a targeted gene disruption of the gene encoding RANTES, donor strains and control recipients (BALB/c, C57BL/6, B6/129, B6.C-H2(bm12)/KhEg(bm12) , H-2^b) were also obtained from Jackson Laboratory. BALB/c differ from C57BL/6 and B6/129 at both class I and class II major histocompatibility complex (MHC) loci, and bm12 mice differ from C57BL/6 and B6/129 at class II alone.

Mouse cardiac allografting (Mottram, P.L. *et al.*, *Transplantation 59*:559-565 (1995); Hancock, W.W., *et al.*, *Proc. Natl. Acad. Sci (USA), 93*:13967-13972 (1996)) was performed with the aid of an operating microscope (Nikon, 4x to 38x magnification) under clean conditions.

Preparation of the donor heart. Donor mice were anesthetized with Nembutal (50 mg/10 g body weight) and Atropine sulfate (0.17 mg/100 g body weight) i.p.; additional anaesthesia with Methoxyflurane supplementation was administered via a face mask as required during the procedure. Mice were shaved and cleansed with 70% alcohol. A midline abdominal incision was made in the donor animal and 1 ml of a 10% solution of heparin in saline was injected into the inferior vena cava. The incision was then extended cephalic to open the chest through a median sternotomy. The thorax was opened. The inferior vena cava was ligated with 6-0 silk and divided inferior to the tie. The superior vena cava was then similarly ligated and divided superior to the tie. The aorta and pulmonary artery were separated and divided as far distally as possible. At this point, blood was evacuated from the heart by applying pressure with applicator sticks. The aorta was transected just proximal to the brachiocephalic artery and the main pulmonary artery transected just proximal to its

bifurcation. The pulmonary veins were then ligated and divided en mass and the heart placed in iced saline.

Preparation of the recipient. After being anesthetized in the same way as the donor, the recipient was brought under the microscope, a midline abdominal incision was made, and segments of the aorta and vena cava below the renal vessels were dissected free, but not separated from each other, over a length of about 2 mm. A clamp was placed on the proximal aorta and vena cava, and a distal tie of 6-0 silk was placed around both the aorta and vena cava in preparation for later occlusion of the vessels.

Transplantation of the heart. The tie that had been placed around the distal aorta and vena cava was secured by means of a single knot. An aortotomy and a venotomy in the vena cava were made adjacent to one another. The donor heart was then removed from the chilled saline, and the donor aorta and pulmonary artery were joined end-to-side to the recipient aorta and vena cava, respectively, with running suture, using 10-0 tipped with a BV-3 needle. Since the anastomoses were done adjacent to one another, the side of the pulmonary artery-cava suture line next to the aortic anastomosis was sutured from the inside with an everting running suture. During this period, chilled saline was dripped on the ischemic heart at frequent intervals. After the completion of the anastomoses, the inferior vascular occluding tie was released first, thus filling the inferior vena cava and donor pulmonary artery with recipient venous blood. Upon release of the proximal occluding tie, the aorta and coronary arteries of the transplant were perfused with oxygenated recipient blood. Blood loss was minimized by gradual release of the proximal tie. Warm saline was used externally to warm the heart immediately after establishing coronary perfusion. With warming and coronary perfusion, the heart began to fibrillate and usually within a few minutes it reverted spontaneously to a sinus rhythm. Occasionally, cardiac massage was required to re-establish a normal beat. The intestines were placed carefully back into the abdominal cavity around the auxiliary heart, and the abdomen was closed with a single running suture to all layers (saline with antibiotic was used to wash the peritoneal cavity as needed). The mouse was then placed in a constant temperature at 35°C for recovery from anesthesia.

Therapeutic intervention. A rat anti-mouse CCR5 monoclonal antibody (IgG2c) was produced by immunizing rats with transfected cells expressing mouse CCR5 followed by fusion of spleenocytes to myeloma cells to produce hybridomas. The anti- mouse CCR5 mAb inhibits chemotaxis of cells expressing recombinant CCR5 upon exposure to the CCR5 ligand MIP-1β, but does not inhibit chemotaxis induced by chemokines which do not bind CCR5. Nonspecific rat IgG was used as a control. The antibodies were administered (200 μg by intraperitoneal injection) to recipient mice at transplantation and every 48 hours thereafter for fourteen days.

Monitoring of allograft survival. Cardiac allograft survival was monitored twice daily by palpation of ventricular contractions through the abdominal wall (Mottram, P.L. *et al., Transplantation, 59*:559-565 (1995)), rejection was defined as the day of cessation of palpable heartbeat, and was verified by autopsy (Gerard, C, *et al., J. Clin Invest., 100*:2022-2027 (1997); Mottram, P.L. *et al., Transplantation, 59*:559-565 (1995)). Once cardiac graft function ceased, mice were anesthetized as above, and grafts were surgically excised, subdivided into portions for (a) formalin fixation, paraffin embedding and subsequent light microscopy examination, or (b) snap-frozen in liquid nitrogen and stored at -70°C until processed for immunohistology or RNAse protection assays.

Immunopathology. For histology, paraffin sections were stained with hematoxylin and eosin (H&E) to evaluate graft morphology, and with Weigert's elastin stain so as to examine the extent of intimal proliferation in penetrating branches of myocardial arteries (a key feature of transplant arteriosclerosis) (Gerard, C, *et al., J. Clin Invest. 100*:2022-2027 (1997); Mottram, P.L., *et al., Transplantation 59*:559-565 (1995)). Chemokine and chemokine receptor mRNA expression was determined using RNAse protection assay kits (Pharmingen, San Diego, CA).

Results

Allograft survival data (mean ± SD) are summarized in Table 1 (using 6-10 animals/group).

Table 1. Effect of inhibition of CCR5 function on mouse cardiac allograft survival

#	Strains (Donor → Recipient)	MHC mismatch	Therapy	Survival (Mean ± SD, days)	probability
1	BALB/c → C57BL/6	class I & II	-	7.3 ± 0.5	
2	BALB/c → CCR5 KO	class I & II	-	21.9 ±1.1	p<0.01 (cf. #1)
3	BALB/c → MIP-1α KO	class I & II	-	7.1 ± 0.9	
4	BALB/c → RANTES KO	class I & II	-	7.4 ± 0.6	
5	bm12 → C57BL/6	class II	-	32.5 ± 2.2	
6	bm12 → CCR5 KO	class II	-	>100	p<0.001 (cf. #5)
7	BALB/c → C57BL/6	class I & II	rat IgG	7.4 ± 0.4	
8	BALB/c → C57BL/6	class I & II	rat anti-mouse CCR5	16.1 ± 0.3	p<0.01 (cf. #6)

p values were determined by the Mann-Whitney U test.

The results presented in Table 1 demonstrate that $CCR5^+$ cells contribute to the pathogenesis of allograft rejection. Disruption of CCR5 function in a complete MHC mismatch significantly prolonged allograft survival (compare groups 1 and 2). In addition, the administration of a neutralizing mAb which binds CCR5 and inhibits the binding of ligand to the receptor also extended the period of allograft survival (compare groups 7 and 8). In contrast, the period of allograft survival was not extended in recipient mice which do not produce MIP-1α or RANTES (groups 3 and 4). These results emphasize the importance of the CCR5 pathway in graft rejection and demonstrate that disruption of receptor function was more effective at preventing rejection than inhibition of individual ligands (compare groups 3 and 4 with groups 2 and 8). Disruption of CCR5 function in class II-mismatch combinations is highly efficacious. Whereas untreated recipients rejected their allografts at around day 33 (group 5), disruption of CCR5 function in this combination resulted in permanent engraftment (group 6).

Pathologic findings

In a further study, hearts from BALB/c donors were grafted into B6/129 recipients. Cardiac allografts in the BALB/c → B6/129 combination using wild type recipients or CCR5 KO recipients were removed after seven days, sectioned, stained (H&E) and studied. Severe rejection was evident in grafts removed from wild type recipients and from wild type recipients that were treated with nonspecific rat IgG. In contrast, grafts removed from CCR5 KO recipients were histologically normal. In addition, treatment of wild type recipients with neutralizing anti-CCR5 mAb led to a preservation of the myocardium and the grafts contained only a mild mononuclear cell infiltrate. Rejection developed after about 16 days in anti-CCR5 mAb treated wild type recipients and after about 22 days in CCR5 KO recipients.

CCR5 KO mice do not have a general defect in cellular immunity and mount normal T cell responses in response to mitogen or antigen (e.g., mixed lymphocyte response).

In further studies, an additional anti-CCR5 monoclonal antibody was characterized and administered to mice that received cardiac allografts that were disparate at both class I and class II MHC loci. This rat anti-mouse CCR5 monoclonal antibody (clone C34-3448, IgG2c, Pharmingen, San Diego, CA, catalog number 559921) was generated using a process that included immunizing a rat with a peptide consisting of amino acids 9-30 of mouse CCR5 that was conjugated to keyhole limpet hemocyanin (KLH).

Competitive ligand binding assays were performed as described herein and as previously described (Topham, P.S. *et al.*, *J. Clin. Invest. 104*:1549-1557 (1999)) to characterize this rat anti-mouse CCR5 monoclonal antibody (clone C34-3448). Briefly, mMIP-1α, mMIP-1β and mRANTES were radiolabeled with ^{125}I and 0.05 nM of the radio-labeled ligand were incubated with 10^5 cells with or without increasing concentrations of rat anti-mouse CCR5 monoclonal antibody (clone C34-3448) or control IgG2c monoclonal antibody. The cells were washed and cell-associated radioactivity was determined in a scintillation counter. Nonspecific

binding was assessed using 100 nM of unlabeled chemokine and specific binding was obtained by subtracting nonspecific binding from total binding (KaleidaGraph®, Synergy Software, Reading, PA). Ligand binding assays were also performed using activated T cells (T cell blasts) which were generated by activation with Concanavalin A (Con-A, Sigma, St. Louis, MO).

The results of the ligand binding assays revealed that anti-CCR5 monoclonal antibody (clone C34-3448) inhibited the binding of the CCR5 ligands mMIP-1α (IC_{50} 1.7 nM), mMIP-1β (IC_{50} 1.4 nM) and mRANTES (IC_{50} 0.2 M), to L1.2 cells expressing recombinant murine CCR5 (CCR5 transfectants) in a dose-dependent manner. The anti-CCR5 monoclonal antibody (clone C34-3448) also inhibited the binding of mMIP-1α (IC_{50} 1.6 nM), mMIP-1β (IC_{50} 1.5 nM) and mRANTES (IC_{50} 0.3 M) to activated T cells (T cell blasts) isolated from CCR5 +/+ mice.

Chemotaxis assays were performed as described herein and as previously described (Topham, P.S. *et al., J. Clin. Invest. 104*:1549-1557 (1999)) to further characterize this rat anti-mouse CCR5 monoclonal antibody (clone C34-3448). Briefly, 10^6 cells in 100 μL of chemotaxis medium (50% M199, 50% RPMI-1640 and 0.5% BSA) were dispensed into the Transwell in the chemotaxis chamber and 600 μL of chemotaxis medium with or without chemokine was placed in the bottom well. The cultures were incubated overnight at 37°C and the number of cells that migrated to the bottom well were counted by flow cytometry. Relative cell counts were obtained by acquiring events for a set time of 30 seconds. Statistical analysis was performed using the Mann-Whitney test.

The results of the chemotaxis assays revealed that anti-CCR5 monoclonal antibody (clone C34-3448) inhibited chemotaxis of L1.2 cells that expressed recombinant murine CCR5 (CCR5 transfectants) or activated T cells (T cell blasts) isolated from CCR5 +/+ mice, in response to the CCR5 ligands mMIP-1α, mMIP-1β or mRANTES. In contrast to these results from CCR5 +/+ mice, no staining by flow cytometry was observed using the anti-CCR5 monoclonal antibody (clone C34-3448) and resting cells or T cell blasts from CCR5 -/- mice.

The results of the ligand binding and chemotaxis assays clearly indicate that rat anti-mouse CCR5 monoclonal antibody (clone C34-3448) binds murine CCR5 and inhibits binding of MIP-1α, MIP-1β and RANTES to the receptor.

This anti-CCR5 monoclonal antibody (clone C34-3448) was administered to CCR5 +/+ (C57BL/6) mice that received BALB/c cardiac allografts (disparate at both class I and class II MHC loci). Rat anti-mouse CCR5 monoclonal antibody (clone C34-3448) or control rat IgG2c (Pharmingen, catalog number 11171S) was administered to recipient mice (200 μg/day, intraperitoneal injection) for 14 days, beginning on the day of transplantation.

Allograft survival data (mean ± SD) depicting the effect of treatment of CCR5 +/+ allograft recipients with rat anti-mouse CCR5 monoclonal antibody (clone C34-3448) or control rat IgG2c antibody (Pharmingen, catalog number 11171S) are summarized in Table 2 (using 6-10 animals/group).

Table 2. Effect of treatment of CCR5 +/+ allograft recipients with rat anti-mouse CCR5 monoclonal antibody (clone C34-3448) or control rat IgG2c.

#	Strains (Donor → Recipient)	MHC mismatch	Therapy	Survival (Mean ± SD, days)	probability
9	BALB/c → C57BL/6 (CCR5 +/+)	class I & II	-	7.5 ± 1.1	
10	BALB/c → C57BL/6 (CCR5 -/-)	class I & II	-	22.8 ± 1.5	p<0.001 (cf. #9)
11	BALB/c → C57BL/6 (CCR5 +/+)	class I & II	rat IgG2c control mAb	8.2 ± 1.0	
12	BALB/c → C57BL/6 (CCR5 +/+)	class I & II	rat anti-mouse CCR5 mAb clone C34-3448	18.2 ± 1.4	p<0.001 (cf. #11)

p values were determined by the Mann-Whitney U test.

Administration of anti-CCR5 monoclonal antibody (clone C34-3448) significantly extended the period of survival of cardiac allografts that were mismatched at both MHC class I and MHC class II loci (compare groups 11 and 12). These results are similar to the results obtained when an anti-CCR5 monoclonal antibody, which was raised against transfected cells that expressed mouse CCR5, was administered to allograft recipient mice (Table 1, groups 7 and 8). The somewhat shorter survival of allografts in recipients treated with anti-CCR5 monoclonal antibody (clone C34-3448), as compared to CCR5 -/- recipients (compare groups 12 and 10), was consistently associated with production of mouse anti-rat antibodies by the recipient mouse at 10-14 days post-transplant.

These allograft studies clearly indicate that disruption of CCR5 function (e.g., by administration of a CCR5 antibody antagonist) significantly prolongs allograft survival.

Example 2 CCR5 and Chronic Rejection in Cardiac Allograft Recipients

The pathogenesis of chronic rejection is a complex process involving accelerated arteriosclerosis (e.g., atherosclerosis) of the graft-associated vasculature and leukocyte infiltration. The effect of disrupting CCR5 function on the development of chronic rejection was assessed by monitoring cardiac allograft survival in CCR5 -/- or CCR5 +/+ recipients that received a low dose of cyclosporin A therapy.

Methods

Mouse cardiac allografting. Cardiac allografts derived from BALB/c donors were transplanted into CCR5 -/- (C57BL/6) recipients or CCR5 +/+ (C57BL/6) control recipients as described in Example 1.

Therapeutic Intervention. The effect of a low dose of cyclosporin A (CsA, Sigma, St. Louis, MO) therapy in CCR5 -/- or CCR5 +/+ allograft recipients was tested by injecting recipient mice with CsA dissolved in olive oil, daily (10 mg/kg/day intraperitoneal injection) until rejection or for a maximum of 14 days, beginning on the day of transplantation.

Histological analysis. For histology, paraffin sections were stained with hematoxylin and eosin (H&E) to evaluate graft morphology, and with Weigert's elastin stain so as to examine the extent of intimal proliferation in penetrating branches of myocardial arteries (a key feature of transplant arteriosclerosis) (Gerard, C, *et al., J. Clin Invest. 100:*2022-2027 (1997); Mottram, P.L., *et al., Transplantation 59:*559-565 (1995)). Cardiac grafts were removed from CCR5 -/- recipients which had been treated with cyclosporin A (group 14) at 100 days post transplant for analysis. All intramyocardial arteries were scored for the extent of intimal proliferation as <5% occlusion (0); 5-20% occlusion (1); 21-40% occlusion (2); 41-60% occlusion (3); 61-80% occlusion (4); or 81-100% occlusion (5) (Murphy *et al., Transplantation 64*:14-19 (1997) and Gao *et al., J. Clin. Invest. 105(1)*:35-44 (2000), the entire teachings of both of which are incorporated herein by reference). Scoring values are representative of 4 grafts/group.

Results

Allograft survival data for CCR5 +/+ and CCR5 -/- recipients which either received or did not receive a low dose of cyclosporin A, are summarized in Table 3 (using 6-10 animals/group).

Table 3 Effect of cyclosporin A treatment on mouse cardiac allograft survival in CCR5 +/+ and CCR5 -/- mice

#	Strains (Donor → Recipient)	MHC mismatch	Therapy	Survival (Mean ± SD, days)	probability
13	BALB/c → C57BL/6 (CCR5 +/+)	class I & II	-	7.5 ± 1.1	
14	BALB/c → C57BL/6 (CCR5 +/+)	class I & II	low CsA	10 ± 1.0	
15	BALB/c → C57BL/6 (CCR5 -/-)	class I & II	-	22.8 ± 1.5	p<0.001 (cf. #13)
16	BALB/c → C57BL/6 (CCR5 -/-)	class I & II	low CsA	>100	p<0.001 (cf. #14)

p values were determined by the Mann-Whitney U test.

The results presented in Table 3 demonstrate that a brief course of cyclosporin A (CsA, 10 mg/kg/d) treatment following transplantation induced only a minor prolongation of allograft survival in CCR5 +/+ control mice (about 2-3 days) as compared with untreated recipients (compare groups 13 and 14). However, the same dose of cyclosporin A given to CCR5 -/- mice (for a maximum of 14 days) led to permanent engraftment in all recipients (group 14). The beneficial actions of some experimental agents can be undermined by concomitant immunosuppression (Smiley, S.T. *et al.*, *Transplantation 70*:415-419 (2000)). However, cyclosporin A (CsA) and inhibition of CCR5 function are synergistic in efficacy.

Histological examination and scoring of blood vessels within grafts removed from cyclosporin A-treated CCR5 -/- allograft recipients after 100 days revealed normal myocardial architecture with an absence of graft infiltration, interstitial fibrosis or development of transplant arteriosclerosis (mean vessel score was 0.2 ± 0.1, n=45 cross-sections of elastin-stained arteries). These findings are in contrast to the severe arteriosclerosis observed in grafts removed from wild type (CCR5+/+) allograft recipients treated with a high dose of cyclosporin A (CsA, 30 mg/kg/d) or CD4 mAb therapy (Mottram, P.L., Han, W. R., *et al.,* "Increased expression of IL-4 and IL-10 and decreased expression of IL-2 and IFN-γ in long-surviving mouse heart allografts after brief CD4-monoclonal antibody therapy," *Transplantation 59:*559-565 (1995); Hancock, W.W., Buelow, R., *et al.,* "Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes," *Nature Medicine 4:* 1392-1396 (1998), the teachings of both of which are incorporated herein by reference).

Additional histological studies of bm12 allografts removed from CCR5 -/- recipients (Table 1, group 6) after 100 days revealed normal myocardial architecture with no evidence of transplant arteriosclerosis or interstitial fibrosis (mean vessel score was 0.2 ± 0.1, n=47 cross-sections of elastin-stained arteries).

These results demonstrate that inhibition of CCR5 function inhibits the development of transplant-associated arteriosclerosis and the development of other features of chronic rejection.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details can be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A method of inhibiting graft rejection comprising administering to a subject in need thereof an effective amount of an antagonist of CCR5 function.

2. The method of Claim 1, wherein said graft is an allograft.

3. The method of Claim 2, wherein said allograft is selected from the group consisting of kidney, liver, lung, heart-lung, pancreas, bowel and heart.

4. The method of Claim 3, wherein said allograft is a heart.

5. The method of Claim 1, wherein said antagonist of CCR5 function is selected from the group consisting of small organic molecules, natural products, peptides, proteins and peptidomimetics.

6. The method of Claim 5, wherein said antagonist of CCR5 function is a small organic molecule.

7. The method of Claim 5, wherein said antagonist of CCR5 function is a natural product.

8. The method of Claim 5, wherein said antagonist of CCR5 function is a peptide.

9. The method of Claim 5, wherein said antagonist of CCR5 function is a peptidomimetic.

10. The method of Claim 5, wherein said antagonist of CCR5 function is a protein.

11. The method of Claim 10, wherein said protein is an anti-CCR5 antibody or antigen-binding fragment thereof.

12. The method of Claim 1, wherein the graft has reduced capacity to express a ligand for CCR5.

13. A method of inhibiting graft rejection comprising administering to a subject in need thereof an effective amount of an antagonist of CCR5 function and an effective amount of an immunosuppressive agent.

14. The method of Claim 13, wherein said immunosuppressive agent is one or more agents selected from the group consisting of calcineurin inhibitors, glucocorticoids, nucleic acid synthesis inhibitors, and antibodies which bind to lymphocytes or antigen-binding fragments thereof.

15. The method of Claim 14, wherein said immunosuppressive agent is a calcineurin inhibitor.

16. The method of Claim 15, wherein said calcineurin inhibitor is cyclosporin A.

17. The method of Claim 15, wherein said calcineurin inhibitor is FK-506.

18. The method of Claim 14, wherein said immunosuppressive agent is a glucocorticoid.

19. The method of Claim 18, wherein said glucocorticoid is prednisone or methylprednisolone.

20. A method of inhibiting graft versus host disease comprising administering an effective amount of an antagonist of CCR5 function to a recipient of a transplanted graft.

21. The method of Claim 20, wherein said graft is bone marrow.

22. The method of Claim 21, further comprising administering an effective amount of an immunosuppressive agent.

23. The method of Claim 22, wherein said immunosuppressive agent is a calcineurin inhibitor.

24. The method of Claim 23, wherein said calcineurin inhibitor is cyclosporin A or FK-506.

25. A method of inhibiting chronic rejection of a transplanted graft comprising administering to a subject in need thereof an effective amount of an antagonist of CCR5 function.

26. The method of Claim 25, wherein said graft is an allograft.

27. The method of Claim 26, wherein said allograft is selected from the group consisting of kidney, liver, lung, heart-lung, pancreas, bowel and heart.

28. The method of Claim 27, wherein said allograft is a heart.

29. The method of Claim 25, wherein said antagonist is an antibody or antigen-binding fragment thereof which binds CCR5.

30. The method of Claim 29, wherein said antibody or antigen-binding fragment thereof binds CCR5 and inhibits the binding of a ligand to CCR5.

31. The method of Claim 25, further comprising administering to said subject an effective amount of an immunosuppressive agent.

32. The method of Claim 31, wherein said immunosuppressive agent is one or more agents selected from the group consisting of calcineurin inhibitors, glucocorticoids, nucleic acid synthesis inhibitors and antibodies which bind to lymphocytes or antigen-binding fragments thereof.

33. The method of Claim 32, wherein said immunosuppressive agent is a calcineurin inhibitor.

34. The method of Claim 33, wherein said calcineurin inhibitor is cyclosporin A.

35. The method of Claim 33, wherein said calcineurin inhibitor is FK-506.

36. The method of Claim 32, wherein said immunosuppressive agent is a glucocorticoid.

37. The method of Claim 36, wherein said glucocorticoid is prednisone or methylprednisolone.

Applicant's or agent's file reference 1855.2006002	International application No.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 13-14, line 23-28, 29-31; and 1-3, respectively

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection (ATCC)

Address of depositary institution *(including postal code and country)*

10801 University Blvd.
Manassas, Virginia 20110-2209
United States of America

Date of deposit	Accession Number
October 25, 1996* and June 6, 1997**	HB-12222* and HB-12366**

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☒

In respect of those designations for which a European patent is sought, the Applicant(s) hereby informs the International Bureau that the Applicant wishes that, until the publication of the mention of the grant of a European patent or for 20 years from the date of filing if the application is refused or withdrawn or deemed to be withdrawn, the biological material deposited with the American Type Culture Collection under Accession No. see * and **

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer: Virginia L Lilly	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(Additional Sheet)

C. ADDITIONAL INDICATIONS (Continued)

shall be made available as provided in Rule 28(3) EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

In respect of the designation of Australia in the subject PCT application, and in accordance with Regulation 3.25(3) of the Australian Patents Regulations, the Applicant hereby gives notice that the furnishing of a sample of the biological material deposited with the American Type Culture Collection under Accession No. see * & ** shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention and who is nominated in a request for the furnishing of a sample.

In respect of the designation of Canada in the subject PCT application, the Applicant hereby informs the International Bureau that the Applicant wishes that, until either a Canadian patent has been issued on the basis of an application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the Commissioner of Patents only authorizes the furnishing of a sample of the biological material deposited with the American Type Culture Collection under Accession No. see *&** and referred to in the application to an independent expert nominated by the Commissioner.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/12206

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 31/00
US CL : 514/1, 885
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/1, 885; 424/85.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,919,776 A (HAGMANN et al.) 06 July 1999 (06.07.1999), see entire document, especially columns 8 and 10 and Claim 1.	1-6, 12-28 and 31-37
Y	US 6,013,644 A (MILLS et al.) 11 January 2000 (11.01.2000), see entire document, especially columns 18-20.	1-6, 12-28 and 31-37
Y	US 6,024,957 A (LAZAROVITS et al.) 15 February 2000 (15.02.2000), see entire document, especially Claims and columns 10-11.	1-6, 12-28 and 31-37

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
24 May 2001 (24.05.2001)

Date of mail[illegible]e international search report
30 AUG 2001

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Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/12206

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-5 (in part), 6, 12-28 (in part) and 31-37 (in part)

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/12206

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-5 (in part), 6, 12-28 (in part) and 31-37 (in part), drawn to a method of inhibiting graft rejection comprising administering an antagonist of CCR5 function, wherein the antagonist is a *small organic molecule*.

Group II, claims 1-5 (in part), 7, 12-28 (in part) and 31-37 (in part), drawn to a method of inhibiting graft rejection comprising administering an antagonist of CCR5 function, wherein the antagonist is a *natural product*.

Group III, claims 1-5 (in part), 8, 12-28 (in part) and 31-37 (in part), drawn to a method of inhibiting graft rejection comprising administering an antagonist of CCR5 function, wherein the antagonist is a *peptide*.

Group IV, claims 1-5 (in part), 9, 12-28 (in part) and 31-37 (in part), drawn to a method of inhibiting graft rejection comprising administering an antagonist of CCR5 function, wherein the antagonist is a *peptidomimetic*.

Group V, claims 1-5 (in part), 10 (in part), 11, 12-28 (in part), 29-30 and 31-37 (in part), drawn to a method of inhibiting graft rejection comprising administering an antagonist of CCR5 function, wherein the antagonist is an *antibody or antigen binding fragment thereof*.

Group VI, claims 1-5 (in part), 10 (in part), 12-28 (in part) and 31-37 (in part), drawn to a method of inhibiting graft rejection comprising administering an antagonist of CCR5 function, wherein the antagonist is a *non-antibody protein*.

The inventions listed as Groups I-VI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions listed as Groups 1-6 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of groups 1-6 is considered to be a method of inhibiting graft rejection by administering an antagonist of CCR5. The invention of Groups 1-6 were found to have no special technical feature that defined the contribution over the prior art of Hagmann et al. (US Pat. No. 5,919,776, see entire document).

Hagmann et al. teach a method of inhibiting graft rejection (e.g., column 10, especially lines 4-24) by administering an antagonist of chemokine receptor function, including CCR5 (e.g., claim 1 and e.g., column 8, especially lines 52-57).

Since Applicant's inventions do not contribute a special technical feature when viewed over the prior art they do not have a single general inventive concept and so lack unity of invention.

Continuation of B. FIELDS SEARCHED Item 3: WEST, MEDLINE[] Search Terms: Inventor name, CCR5, allograft, graft, transplantation, chemokine